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In the neuronal cell line SH-SY5Y, oxidative stress-induced free radical overproduction causes cell death without any participation of intracellular Ca^{2+} increase

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Abstract

Adding the membrane-permeant oxidant tert-butylhydroperoxide (t-BOOH) to the incubation medium, in SH-SY5Y human neuroblastoma cells, induced a marked and progressive concentration-dependent (300, 500 and 1000 μM) increase of free radical production, as evaluated by the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and of the intracellular Ca^{2+} ion concentrations $[Ca^{2+}]_i$. The removal of extracellular Ca^{2+} ions did not prevent t-BOOH-induced $[Ca^{2+}]_i$ elevation, whereas the intracellular Ca^{2+} ion chelator 1,2-bis(*o*-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid (BAPTA) (10 μM) was shown to be effective. Both t-BOOH-induced free radical formation and the $[Ca^{2+}]_i$ increase were completely prevented by the peroxyl scavenger α-tocopherol (50 μM). t-BOOH induced a time-dependent SH-SY5Y cell injury, monitored by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (approximately 25% at 1 h, 50% at 3 h, 80% at 5 h) and by fluorescein diacetate (FDA)-propidium iodide (PI) fluorescent staining. The entity of t-BOOH-induced cell damage was the same both in the absence and in the presence of the intracellular Ca^{2+} ion chelator BAPTA. By contrast, the peroxyl scavenger α-tocopherol (50 μM) completely prevented cell injury due to oxidative stress. Finally, superoxide dismutase (SOD) (500 ng/ml) caused a 30% reduction of t-BOOH-induced 2′,7′-dichlorofluorescein (DCF) fluorescence, whereas it did not modify the extent of cell injury produced by the oxidant. Collectively, the results of the present study demonstrated that in SH-SY5Y human neuroblastoma cells, the rise of $[Ca^{2+}]_i$ which occurs during oxidative stress is not involved in cell injury. Therefore, oxidative stress-induced cell death may be exclusively attributed to free radical overproduction.

Keywords: Free radical; Intracellular Ca^{2+} ion concentration; SH-SY5Y; Cell death

Abbreviations: t-BOOH, tert-butylhydroperoxide; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; BAPTA, 1,2-bis(*o*-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; $[Ca^{2+}]_i$, intracellular Ca^{2+} ion concentration; FDA, fluorescein diacetate; PI, propidium iodide; SOD, superoxide dismutase; MDA, malondialdehyde

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1. Introduction

In the last 10 years, a large body of evidence has been accumulated suggesting that the increase of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and/or of free radical formation during oxidative stress are involved in cell death in different cell types. However, the relative contribution and the temporal sequence of these cellular events leading to cell death is still controversial.

In fact, on the one hand, it has been suggested that the increase of $[Ca^{2+}]_i$ induces an exacerbation of oxidative stress [1], compromises mitochondria [2], activates Ca^{2+}-dependent enzymes such as the calpain and the endonuclease pathways which ultimately cause cell injury [3] and disrupts the cytoskeleton [4,5]. All these events play a crucial role in cell death. On the other hand, it has been proposed that the elevation of $[Ca^{2+}]_i$ seems to be only a late phenomenon and is not appreciably involved in oxidant-induced cell death. In this case, free radical overproduction compromises both matrix and membrane mitochondrial enzymes with a subsequent inhibition of ATP synthesis and cell injury [6–9].

In the central nervous system, free radical overproduction seems to be involved in the acute damage caused by cerebral ischemia and in the progressive neuronal damage of chronic diseases such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis. In these disorders, the relative contribution of free radical overproduction and of $[Ca^{2+}]_i$ deregulation in oxidant-induced cell death remains still unclear. In fact, it has been shown that free radical overproduction directly causes apoptosis of immature cultured cortical neurons [10], directly induces DNA damage [11,12] and depletion of the intracellular thiol antioxidant glutathione with a subsequent apoptotic [10] and necrotic cell death [13]. On the other hand, it has also been suggested that free radicals and Ca^{2+} ions cooperate to induce neuronal cell injury. This seems to occur in glutamate-induced neuronal death [14] and also during oxidative stress. In fact, it has been shown that oxidative stress increases $[Ca^{2+}]_i$, which leads to activation of endonucleases that degrade DNA and ultimately contribute to cell death [11,12,15]. Furthermore, it has been demonstrated that free radical overproduction may inhibit Ca^{2+}-ATPases, which leads to an altered regulation of Ca^{2+} levels and cell death [16].

In this study, we investigated in the SH-SY5Y human neuroblastoma cell line whether free radical production during oxidative stress is by itself sufficient to induce cell damage or whether the elevation of $[Ca^{2+}]_i$ may participate in oxidative cell injury. For this purpose, SH-SY5Y neuronal cells were exposed to oxidative stress induced by tert-butylhydroperoxide (t-BOOH), which has been extensively used to induce both free radical overproduction and cytosolic Ca^{2+} increase [17–20], thus allowing us to study the relationship between these two events in the mechanisms leading to cell death. Free radical production was monitored by the evaluation of the fluorescence intensity of 2′,7′-dichlorodihydrofluorescein diacetate [21,22] (DCFH-DA). Lipid peroxidation was determined by assaying the intracellular malondialdehyde (MDA) production by means of the thiobarbituric acid test [23]. $[Ca^{2+}]_i$ was detected by the fluorescent probe FURA-2 [24]. Cell viability was determined by evaluating mitochondrial activity using the ability of living cells to convert soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to insoluble formazan salts [25–27] and by fluorescein diacetate (FDA)-propidium iodide (PI) fluorescent staining [28,29].

2. Materials and methods

2.1. Cell culture

The SH-SY5Y human neuroblastoma cell line was cultured as monolayer in polystyrene dishes and maintained in minimal essential medium (Gibco BRL) containing nutrient mixture F-12 Ham (v/v 1:1), 10% fetal bovine serum (Hyclone, UT, USA), 1% of 200 mM L-glutamine, 1% of 100× non-essential amino acid solution, 100 IU/ml penicillin, 100 μg/ml streptomycin (ICN). Cells were grown in a humidified incubator at 37°C in a 5% CO_2 atmosphere and were fed twice a week. All experiments were performed in multiple well flow dishes.

2.2. Determination of free radical production and lipid peroxidation

Free radical production was measured by incubating the SH-SY5Y human neuroblastoma cells in the presence of DCFH-DA [21,22] (Molecular Probes, Irvine, CA, USA). DCFH-DA is a stable, non-fluorescent molecule that readily crosses the cell membrane and is hydrolyzed by intracellular esterases to the non-fluorescent probe DCFH, that is rapidly oxidized in the presence of peroxides to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). Cells (2×10^6/ml) were loaded with 1 μM DCFH-DA for 30 min at 37°C, in a medium whose composition was in mM: NaCl 138, KCl 2.7, $CaCl_2$ 1.2, $MgCl_2$ 1.2, phosphate-buffered saline (PBS) 10, glucose 10, pH 7.4 (standard medium). After the loading period, cells were washed twice before the experiment was performed. The fluorescence was recorded at 495 nm Ex and 530 nm Em (bandpass 2.5 nm) in a Perkin-Elmer LS 50B spectrophotofluorimeter. Lipid peroxidation was determined by assaying the MDA production by means of the thiobarbituric acid test [23].

2.3. Measurements of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ values were detected by using the fluorescent probe FURA-2 (Calbiochem, San Diego, CA, USA). Briefly, cells (2×10^6/ml) were incubated with 5 μM FURA-2-AM for 45 min at 37°C. After the loading period, the medium was diluted with two volumes of the same balanced salt solution, incubated at 37°C and then washed twice before beginning the experiment. $[Ca^{2+}]_i$ values were measured in a 2 ml cell suspension (1×10^6/ml), in a quartz cuvette equipped with a magnetic stirrer bar. FURA-2 fluorescence was monitored in a Perkin-Elmer model LS 50B spectrophotofluorimeter. The excitation wavelengths were 340 and 380 nm (bandpass 5 nm) with the emission at 510 nm (bandpass 5 nm). $[Ca^{2+}]_i$ values were determined according to the equation of Grynkiewicz et al. [24].

2.4. Determination of cell viability evaluated as mitochondrial activity

Cell viability evaluated as mitochondrial activity was quantified by measuring dehydrogenase activity retained in the cultured cells, using the MTT (Sigma, Italy) assay [25–27]. The assay is based on the ability of living cells to convert dissolved MTT into insoluble formazan. Therefore, the amount of formazan produced is proportional to the number of living cells. Briefly, 2×10^6 SH-SY5Y human neuroblastoma cells were incubated in 2 ml MTT solution (0.5 mg/ml) for 1 h in a humidified 5% CO_2 incubator at 37°C. Thereafter, the medium was removed and cells were washed with PBS. Then, 1 ml of dimethylsulfoxide (DMSO) was added to the cells to solubilize the formazan. The absorbance was read at 540 nm. Data are expressed as the percentage of cell injury to sham-treated cultures.

2.5. Intravital staining of the culture

After the experimental procedures, SH-SH5Y cells (5×10^5/ml/well) were washed with the standard medium and stained for 3 min at 22°C with a solution containing 36 μM FDA (Sigma, Italy) and 7 μM PI (Calbiochem, San Diego, CA, USA). The stained cells were examined immediately with a standard epi-illumination fluorescence microscope. FDA, a non-polar ester, crosses the cell membrane and is hydrolyzed by intracellular esterases to produce a green-yellow fluorescence. Cell injury curtails FDA staining and allows for cell permeation with PI, a polar compound which, by interacting with nuclear DNA, yields a bright red fluorescence [28,29].

2.6. Drugs

t-BOOH (Sigma-Aldrich, Italy) (stock solution 100 mM) was dissolved in H_2O. DCFH-DA (stock solution 10 mM), FURA-2-AM and 1,2-bis(*o*-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid (BAPTA)-AM (stock solution 10 mM) (Calbiochem, San Diego, CA, USA) were dissolved in DMSO. α-Tocopherol (Sigma-Aldrich, Italy,) was solubilized with ethanol (stock solution 100 mM). The final concentrations of DMSO and ethanol in the experimental medium were always less than 1%. Superoxide dismutase (SOD) (Sigma-Aldrich, Italy) was diluted in H_2O. FDA was dissolved in acetone and PI was diluted in water, both were protected from the light.

2.7. Statistics

Data were analyzed by one way analysis of variance followed by the Newmann-Keul's test.

3. Results

3.1. Effect of the peroxyl scavenger α-tocopherol on oxidative stress-induced free radical production

t-BOOH induced a rapid, progressive and dose-dependent (300, 500 and 1000 μM) increase of free radical production in the SH-SY5Y human neuroblastoma cell line (Fig. 1A). α-Tocopherol (50 μM), a peroxyl scavenger which blocks lipid peroxidation by donating an electron to peroxyl radicals, thus converting it to a lipid hydroperoxide that can be decomposed by the enzyme glutathione peroxidase [30], added to the incubation medium 30 min before the membrane-permeant oxidant t-BOOH (1 mM), prevented the free radical formation measured as DCF fluorescence increase (Fig. 1B) and lipid peroxidation measured with a MDA assay (Fig. 1C).

3.2. Effect of α-tocopherol and of the removal of extracellular Ca^{2+} ions or the chelation of intracellular Ca^{2+} by BAPTA on the $[Ca^{2+}]_i$ increase evoked by oxidative stress

The removal of extracellular Ca^{2+} ions did not modify the $[Ca^{2+}]_i$ increase evoked by 1 mM t-BOOH since $[Ca^{2+}]_i$ values were similar both in the presence and in the absence of extracellular Ca^{2+} ions (Fig. 2A). By contrast, the intracellular Ca^{2+} chelator BAPTA [31] (10 μM) completely prevented the oxidative stress-induced $[Ca^{2+}]_i$ increase (Fig. 2B). α-Tocopherol (50 μM), added to the incubation medium 30 min before the membrane-permeant oxidant t-BOOH (1 mM), also prevented $[Ca^{2+}]_i$ increase elicited by oxidative stress (Fig. 2C).

3.3. Effect of α-tocopherol and of the removal of extracellular Ca^{2+} ions or the chelation of intracellular Ca^{2+} by BAPTA on cell injury evoked by oxidative stress

When oxidative stress-induced cell damage was

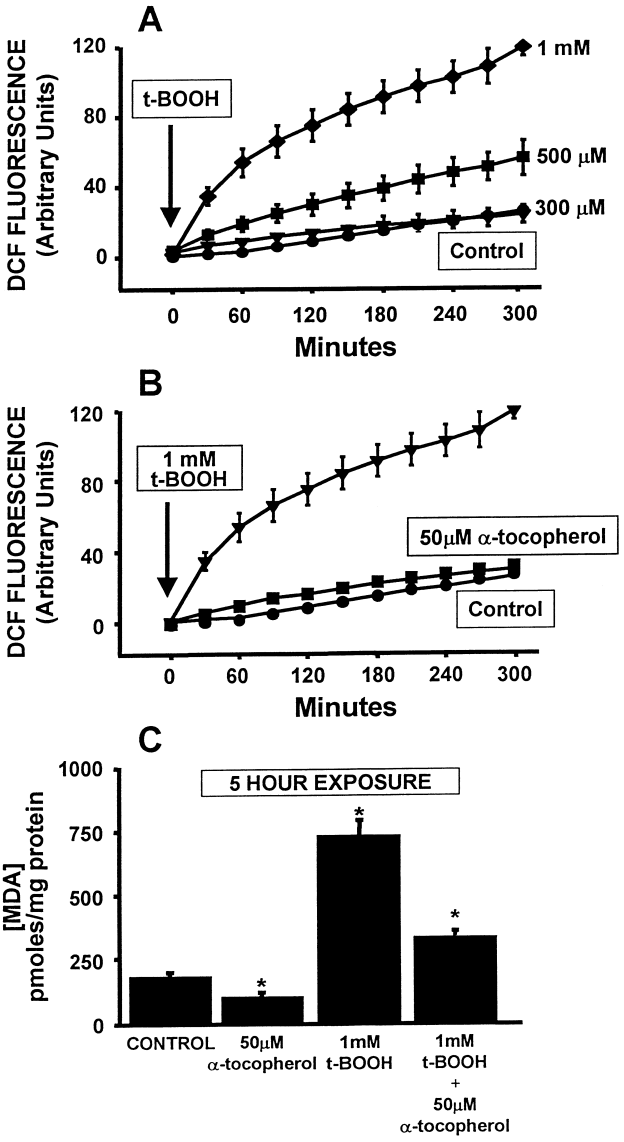

Fig. 1. Effect of the peroxyl scavenger α-tocopherol on oxidative stress-induced free radical production. SH-SY5Y human neuroblastoma cells were loaded with 1 μM DCFH-DA for 30 min at 37°C and then exposed to increasing concentrations of t-BOOH for a 5 h period in the absence (A) or in the presence of 50 μM α-tocopherol (B). The arrow indicates the time at which t-BOOH was added. Fluorescence was measured in arbitrary units, as described in Section 2. Values are the means ± S.E.M. of four separate experiments. (●) indicates control cells loaded with DCFH-DA and not exposed to t-BOOH (A) or to t-BOOH and α-tocopherol (B). C: SH-SY5Y human neuroblastoma cells were exposed to oxidative stress for a 5 h period in the presence or in the absence of the peroxyl scavenger α-tocopherol (50 μM). Each column represents the mean ± S.E.M. of four independent experiments. $^*P < 0.05$ versus all other groups.

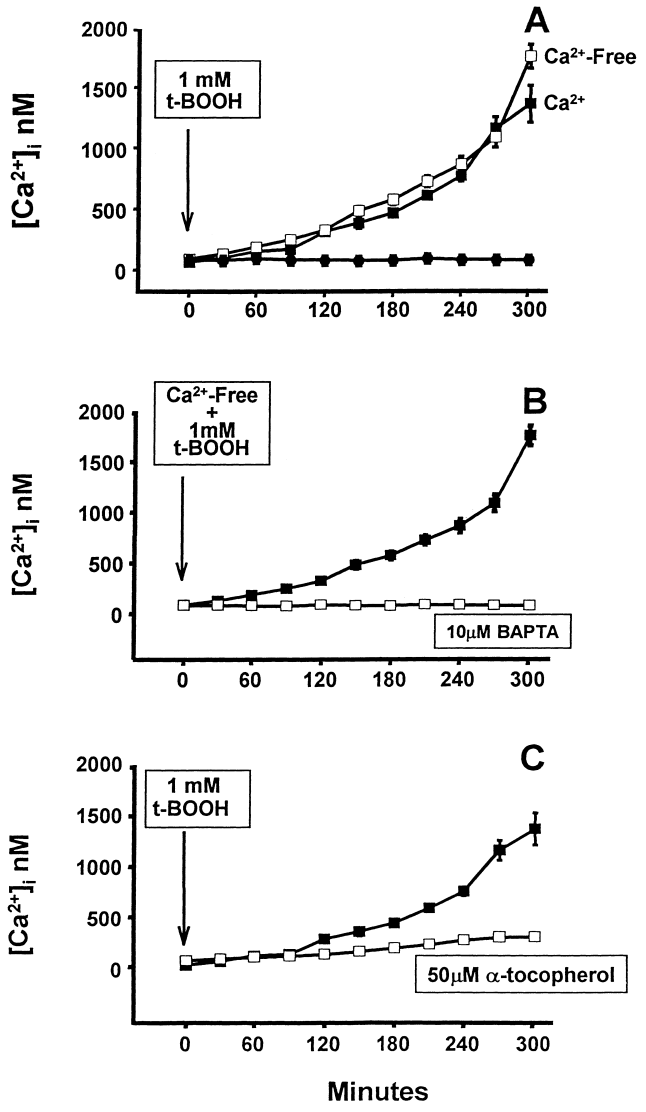

Fig. 2. Effect of α-tocopherol and of the removal of extracellular Ca^{2+} ions or the chelation of intracellular Ca^{2+} by BAPTA on the $[Ca^{2+}]_i$ increase evoked by oxidative stress. SH-SY5Y human neuroblastoma cells were incubated with 5 μM FURA-2-AM for 45 min at 37°C. After washing, the cells were incubated in a standard medium (●) or exposed to 1 mM t-BOOH for a 5 h period in the absence (□) or in the presence (■) of extracellular Ca^{2+} ions (1.2 mM) (A) or in the absence of extracellular Ca^{2+} ions with (□) or without BAPTA-AM (10 μM) (■) (B). BAPTA-AM was pre-incubated for 60 min at 37°C before time 0. The arrow indicates the time at which t-BOOH (1 mM) was added. Values are the means ± S.E.M. of three separate experiments. In C, after washing, the cells were exposed to oxidative stress (1 mM t-BOOH) in the presence (□) or in the absence (■) of 50 μM α-tocopherol. Values are the means ± S.E.M. of three separate experiments.

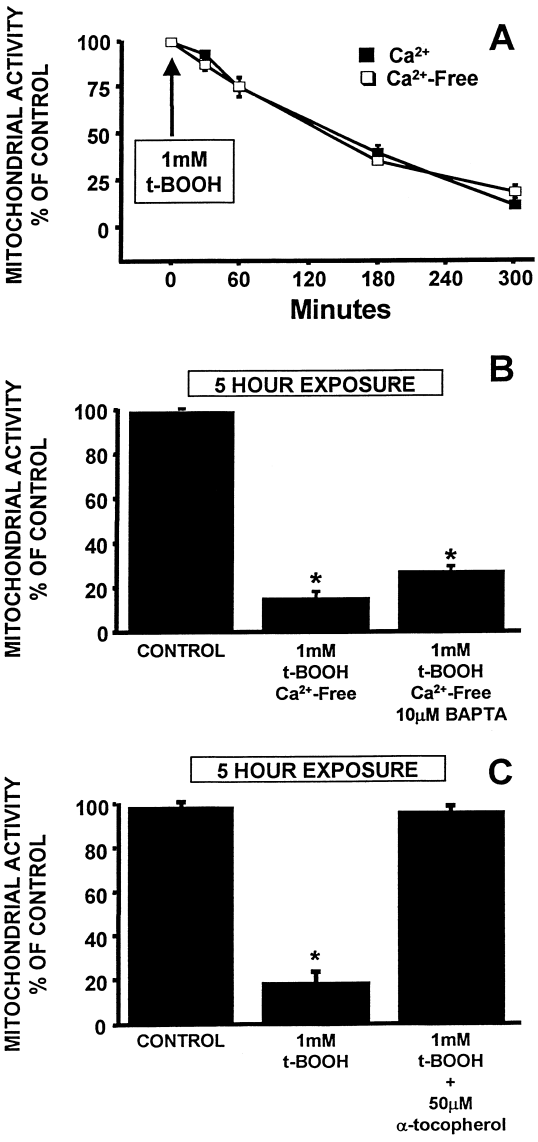

Fig. 3. Effect of α-tocopherol and of the removal of extracellular Ca^{2+} ions or the chelation of intracellular Ca^{2+} by BAPTA on cell injury evoked by oxidative stress. A: SH-SY5Y human neuroblastoma cells were exposed to oxidative stress (1 mM t-BOOH) for 30, 60, 180 and 300 min, respectively, in the presence (■) or in the absence (□) of extracellular Ca^{2+} ions and assessed in their ability to reduce MTT. For experimental details, see Section 2. Each point represents the mean ± S.E.M. of three independent experiments. B: SH-SY5Y human neuroblastoma cells were exposed to oxidative stress (1 mM t-BOOH) for a 5 h period in the absence of extracellular Ca^{2+} ions with or without 10 μM BAPTA. Each column represents the mean ± S.E.M. of three independent experiments. $^*P < 0.05$ versus the control group. C: SH-SY5Y cells were exposed to oxidative stress for a 5 h period in the presence or in the absence of the peroxyl scavenger α-tocopherol; (50 μM). Each column represents the mean ± S.E.M. of three independent experiments. $^*P < 0.05$ versus the control group and versus the 1 mM t-BOOH plus α-tocopherol group.

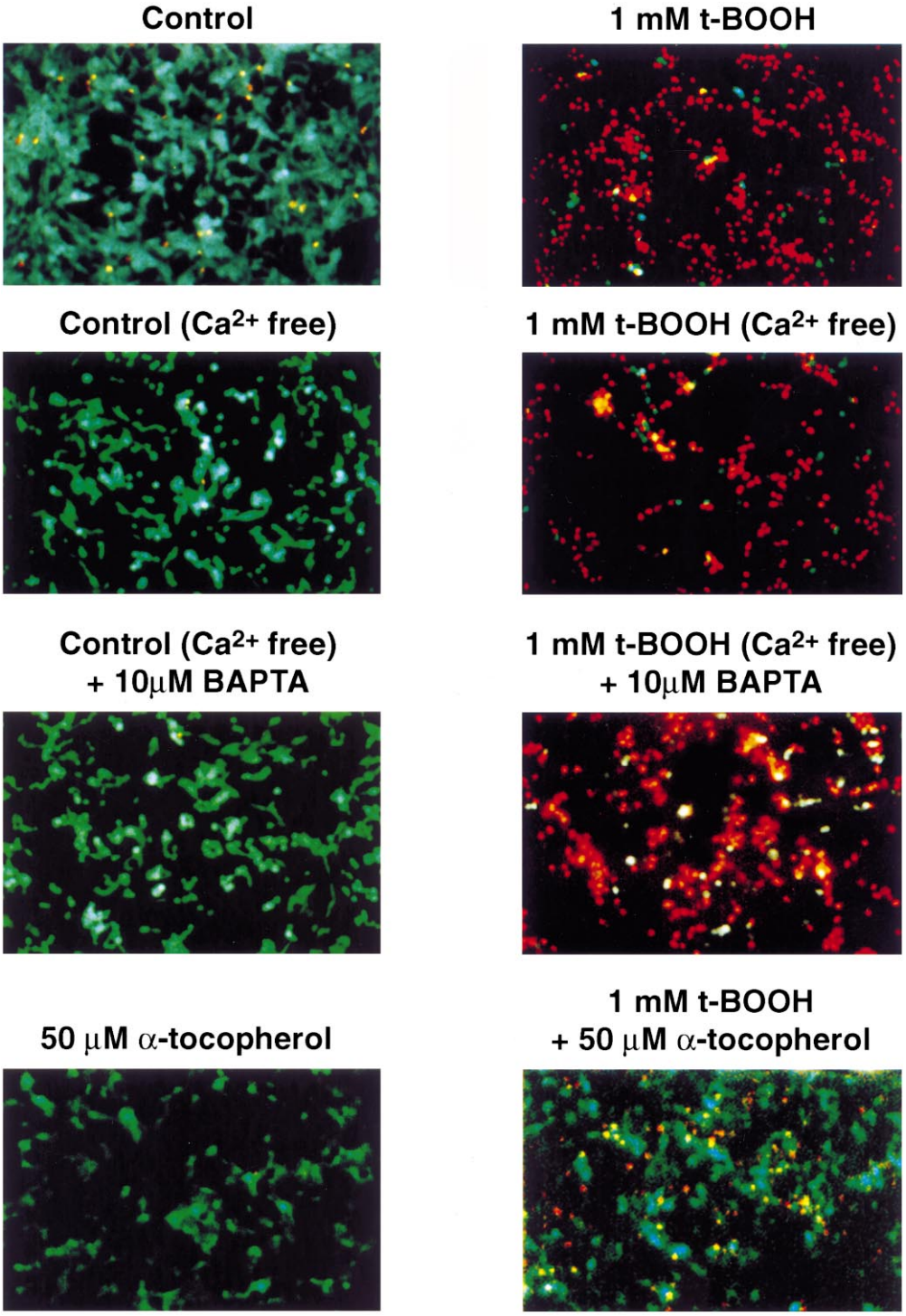

Fig. 4. Effect of the peroxyl scavenger α-tocopherol and of the extracellular Ca^{2+} removal or the intracellular Ca^{2+} chelation by BAPTA on SH-SY5Y cell death induced by oxidative stress. SH-SY5Y human neuroblastoma cells were exposed to oxidative stress (1 mM t-BOOH) for a 5 h period in the presence of extracellular Ca^{2+} ions or in the presence of extracellular Ca^{2+} ions plus 50 μM α-tocopherol, or in the absence of extracellular Ca^{2+} ions or in the absence of extracellular Ca^{2+} ions plus 10 μM BAPTA and then stained for 3 min at 22°C with a solution containing 36 μM FDA and 7 μM PI. The drawing is representative of at least three independent experiments. For experimental details, see Section 2.

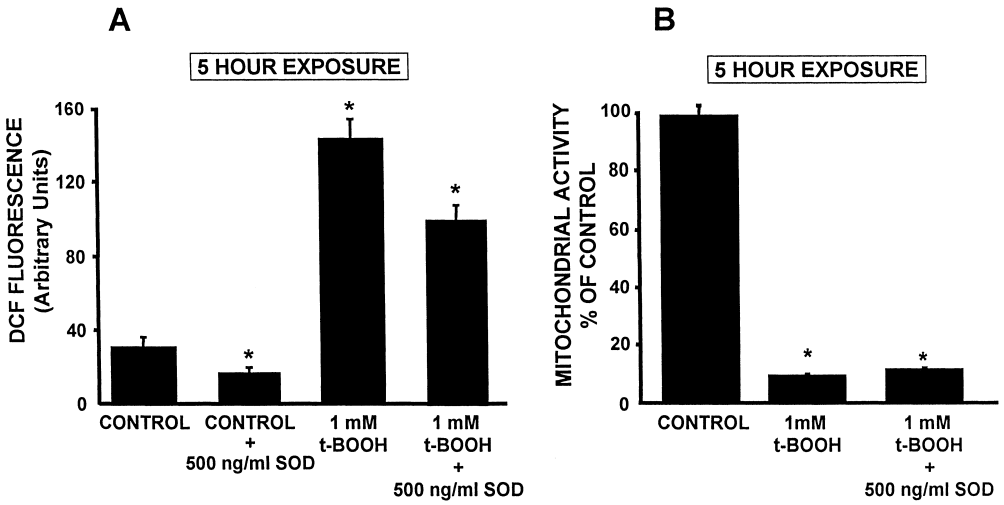

Fig. 5. Effect of SOD on t-BOOH-induced DCF fluorescence increase and cell injury in SH-SY5Y human neuroblastoma cells. A: SH-SY5Y human neuroblastoma cells were loaded with 1 μM DCFH-DA and then exposed to 1 mM t-BOOH for a 5 h period, in the presence or in the absence of 500 ng/ml SOD. Each column represents the mean ± S.E.M. of four independent experiments. *$P < 0.05$ versus all other groups. B: SH-SY5Y human neuroblastoma cells were exposed to oxidative stress (1 mM t-BOOH) for a 5 h period in the presence or in the absence of 500 ng/ml SOD and then assessed in their ability to reduce MTT. *$P < 0.05$ versus the control group.

evaluated, t-BOOH (1 mM) elicited a time-dependent cell injury of SH-SY5Y human neuroblastoma cells measured by the MTT assay and FDA-PI intravital staining. At 1 h, the percentage of cell damage measured as MTT assay was approximately 25%, reaching 50% at 3 h and 80% at 5 h (Fig. 3A). Interestingly, MTT-monitored cell injury induced by free radical elevation was of the same entity, both in the presence and in the absence of extracellular Ca^{2+} ions (Fig. 3A). Similar results were obtained with FDA-PI intravital staining. In fact, extracellular Ca^{2+} removal did not prevent the increase of PI positive SH-SY5Y cells induced by t-BOOH (Fig. 4). Finally, the lowering of $[Ca^{2+}]_i$ induced by the membrane permeable Ca^{2+} chelator BAPTA [31] did not prevent t-BOOH-induced cell damage, measured as a MTT assay (Fig. 3B) or by FDA-PI intravital staining (Fig. 4), after 5 h of exposure. In contrast, when the peroxyl scavenger α-tocopherol (50 μM) was added to the incubation medium 30 min before the membrane-permeant oxidant t-BOOH (1 mM), the injuring effect of oxidative stress, as shown with the MTT assay (Fig. 3C) or with FDA-PI intravital staining (Fig. 4), was completely prevented.

3.4. *Effect of SOD on t-BOOH-induced free radical overproduction and SH-SY5Y cell injury*

SOD (500 ng/ml), an enzyme which specifically scavenges the superoxide radical to molecular oxygen [32], caused a 30% reduction of the t-BOOH-induced DCF fluorescence increase (Fig. 5A), demonstrating that a significant amount of superoxide is produced together with other free radical species following the use of t-BOOH. However, SOD (500 ng/ml) was unable to prevent cell injury induced by oxidative stress (Fig. 5B).

4. Discussion

The results of the present study demonstrated that in a neuronal cell type like the SH-SY5Y human neuroblastoma, the delayed rise of $[Ca^{2+}]_i$ occurring upon exposure to oxidative stress is not involved in cell death. Therefore, cell injury can be only attributed to the overproduction of free radicals.

The exposure of SH-SY5Y cells to the membrane-permeant oxidant t-BOOH caused a rapid increase of

DCF-monitored free radical formation, which only after a long delay elicited a sustained $[Ca^{2+}]_i$ elevation. The hypothesis that free radical production is responsible for a $[Ca^{2+}]_i$ increase is supported by the ability of the peroxyl scavenger α-tocopherol to prevent $[Ca^{2+}]_i$ elevation through the scavenging of free radicals. The increase of the cytoplasmic concentration of the bivalent cation can not be attributed to an influx from the extracellular space since its removal from the incubation medium did not prevent the $[Ca^{2+}]_i$ elevation elicited by t-BOOH. This finding would suggest that, at least in the time interval considered in the present study, free radicals do not produce a plasma membrane damage able to allow for Ca^{2+} influx from the extracellular space. Rather, the $[Ca^{2+}]_i$ increase induced by oxidative stress seems to be the consequence of free radical action on Ca^{2+} ions stored in intracellular organelles. In fact, in the presence of BAPTA-AM, a membrane permeable Ca^{2+} chelator trapped in the cells after its cytoplasmic hydrolization [31], $[Ca^{2+}]_i$ elevation was prevented. All these results are in line with previous findings obtained in different cell types, showing that t-BOOH-induced radical formation precedes the increase in cellular free Ca^{2+} deriving from intracellular sources [18,20,33]. However, Ca^{2+} release from intracellular organelles induced by oxidative stress does not seem to be involved in SH-SY5Y cell injury. In fact, significant SH-SY5Y cell death had already occurred at 1 h, a time when there is a remarkable presence of free radical production and the $[Ca^{2+}]_i$ increase has not yet occurred. This evidence suggests that free radicals interfere directly with cell survival without any participation of Ca^{2+} ions. This is further supported by the results of the experiments performed on cell survival in the presence of the intracellular Ca^{2+} chelator BAPTA after 5 h of oxidative stress, when the maximal injury occurs. In fact, buffering of the elevated $[Ca^{2+}]_i$ obtained with BAPTA did not prevent SH-SY5Y cell injury induced by t-BOOH, whereas the free radical scavenger α-tocopherol completely protected these cells. These results are in contrast with data recently published by Liu et al. (1998), who showed that in the LLC-PK1 cells, chelation of intracellular Ca^{2+} by EGTA-AM prevents t-BOOH-induced cell killing, evaluated at the sixth hour [18]. Although it is difficult to explain these opposite results obtained in rather similar experimental conditions, it is possible that the cell type may play a key role in the mechanisms leading to cell death during oxidative stress. This hypothesis seems to be reasonable if one considers that also in myocytes and in hepatocytes, a $[Ca^{2+}]_i$ increase does not seem to be involved in cell injuring induced by oxidants [34,35]. Whichever mechanism, i.e. $[Ca^{2+}]_i$ increase and/or free radical overproduction, is involved in t-BOOH-induced cell death, this event can be blocked by antioxidants. In fact, the present results clearly showed the protective effect of α-tocopherol in SH-SY5Y neuroblastoma cells exposed to t-BOOH and the same protection by antioxidants was observed in LLC-PK1 cells [36].

A novel finding deserving further discussion is the effect of SOD addition on DCF-monitored free radical production and cell death. The results of the present study showing that the addition of SOD caused a 30% reduction of the t-BOOH-induced DCF fluorescence increase strongly suggests that a significant amount of superoxide is produced together with alkoxyl, methyl and peroxyl radicals [37,38]. However, the presence of SOD did not induce any change in the extent of t-BOOH-induced cell death. These findings seem to suggest that superoxide production may not be involved in t-BOOH-elicited SH-SY5Y cell injury. However, it cannot be excluded that oxidative stress may be sufficient to cause maximal injury even with SOD present or that SOD might poorly enter the cell and may thus be minimally efficient in reducing the intracellular oxidant stress.

In conclusion, the results of the present study show that in SH-SY5Y neuroblastoma cells during t-BOOH-induced oxidative stress, free radical overproduction may induce cell injury without any participation of $[Ca^{2+}]_i$ increase, as, on the contrary, it occurs in other cell types. Therefore, caution must be exerted in drawing general conclusions on the mechanisms underlying oxidant-induced cell death.

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